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TITLE: Levels of the Novel Glycoprotein Lacritin in Human Tears After Laser Refractive Surgery

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14. ABSTRACT
Lacritin is a naturally occurring tear protein with antimicrobial activity that is capable of stimulating mitogenesis in human corneal epithelial cells and promoting production of tears in lacrimal gland acinar cells. Heparanase (HPSE) acts as a regulator for lacritin by cleaving heparan sulfate chains and allowing lacritin to bind. We aim to measure both tear lacritin and HPSE pre- and post-operatively to elucidate lacritin and HPSE's response in patients undergoing PRK (photorefractive keratectomy) and LASIK (Laser-assisted in situ keratomileusis) with the possibility of the development of recombinant lacritin as a novel therapeutic agent for wound healing. Up to 196 patients eligible to undergo PRK or LASIK at the U.S. Army Warfighter Refractive Surgery Research Center at Fort Belvoir will be consecutively recruited: 98 PRK (49 male and 49 female) and 98 LASIK (49 male and 49 female). The purpose of this study is to measure tear lacritin and HPSE levels following surgery using a minimal risk procedure to collect tears from patients undergoing PRK or LASIK. Tears will be collected using a safe and established method that employs the use of a polyester fiber rod (Transorb Wick, Filtrona, Richmond, VA). The wicks will soak up tears from the inferior cul-de-sac of the left eye of each subject at the pre-operative visit and at 1 day, 1 week, 1, 3 and 6 months post-operatively to quantify tear lacritin and HPSE. Study design will allow for within subject comparison of lacritin and HPSE before and after surgery as well as comparison of responses between procedures (PRK vs. LASIK). The primary outcome measure is tear lacritin levels pre- and post-surgery. The secondary outcome is tear HPSE levels pre-and post-surgery.

15. SUBJECT TERMS
Refractive Surgery, PRK, LASIK, lacritin, heparanase, dry eye, optical quality

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INTRODUCTION

PRK is the most commonly performed refractive eye surgery in the US Army while LASIK is the most commonly performed refractive surgery worldwide. PRK involves the creation of a large corneal epithelial defect followed by excimer laser photoablation of the underlying corneal stroma to achieve the desired refractive effect. Healing in the early PRK post-operative period first involves epithelial healing, followed by stromal remodeling. Factors promoting more rapid re-epithelialization of the corneal surface are beneficial in hastening return to full function and reducing the risk of postoperative infection, corneal haze, and scarring. In LASIK, a thin corneal flap is created using either a femtosecond laser. The hinged corneal flap is then temporarily reflected back while the excimer laser is used to reshape the central cornea. After the laser treatment, the corneal flap is floated back into position where it stays in place without the need of sutures. LASIK preserves the anterior layers of the cornea and the resulting healing process involves relatively rapid visual healing with minimal pain. It has a low risk for haze and regression and therefore allows for the treatment of higher degrees of refractive error.

Both LASIK and PRK result in dry eye. Because many military operations are performed in harsh environmental conditions that may exacerbate dry eyes, even mild cases of surgery induced dry eye could prove not only uncomfortable but potentially life-threatening if quality of vision is significantly impacted in the combat environment. Because of the common occurrence of dry eye in post-refractive patients and the relative inability for deployed soldiers to seek routine ophthalmic care, it is important to understand the factors that contribute to dry eye and to explore and develop potential treatments that promise to prevent or alleviate this condition.

Lacritin has several intriguing therapeutic and wound-healing properties. This study pursues lacritin and its regulator HPSE's response to the surgical stress of PRK and LASIK. In the future, we hope to direct recombinant lacritin to improve wound healing, visual outcomes, and dry eye. As lacritin stimulates regeneration of human corneal epithelial cells in vitro, it may promote re-epithelialization following PRK. In doing so, it may foster a more controlled wound healing process thereby contributing to the accuracy of refractive outcomes. Furthermore, because lacritin has been shown to stimulate tear production, treatment with recombinant lacritin may ameliorate or prevent dry eye following laser refractive surgery.

This is a collaborative effort between the U.S. Army Warfighter Refractive Surgery Research Center at Fort Belvoir (WRSRC) previously known as Center for Refractive Surgery (CRS) at Walter Reed Army Medical Center (WRAMC), James Madison University, and the Rappaport Faculty of Medicine.

BODY

With the 2005 Base Realignment and Closure Act (BRAC), Walter Reed Army Medical Center and the National Naval Medical Center in Bethesda merged and formed a new Walter Reed National Military Medical Center (WRNMMC) in the north capital area and Fort Belvoir Community Hospital (FBCH) in the south. In response to the BRAC and personnel changes, the

principal investigator (PI), along with the WRSRC staff, determined that the following modifications would best serve the long term success of the research activities:
-To address personnel changes, a modification was submitted as part of the October 2011continuing review requesting a change in PI from Dr. Michael Mines to Denise Ryan and the removal of investigators no longer participating in the study (**Appendix 1**).
-Due to inadequate temperature and humidity controls in the FBCH laser room, the WRNMMC laser vision center was selected to accommodate treatments. A modification was submitted requesting enrollment, study informed consent, screening, pre- and post-operative eye exams all be conducted at FBCH. All treatments would be conducted at WRNMMC until available at FBCH.
-To accommodate BRAC changes requested by the integrated WRNMMC IRB, the following modifications were submitted:

- A transition document was submitted to the WRNMMC IRB to indicate the course of action and patient communication plan for the study during BRAC.
- Modifications were submitted to update the WRAMC consent form to the current WRNMMC DRP format.

-The currently approved consent form and approval letters for the modifications and continuing reviews from the WRNMMC IRB are attached as **Appendix 1** at the conclusion of this report. The current JMU IRB approval is attached as **Appendix 2.**

-Current enrollment and follow up rates are summarized in **Table 1.**

Table 1: Summary of enrollment and follow up rates:

	Enrolled			**1M**		**3M**		**6M**		**12M**	
Phase I	PRK (M/F)	LASIK (M/F)		PRK (M/F)	LASIK (M/F)	PRK (M/F)	LASIK (M/F)	PRK (M/F)	LASIK (M/F)	PRK (M/F)	LASIK (M/F)
Total required	49/49	49/49	Seen for Visit	44/18	13/6	34/16	12/5	29/14	12/5	26/12	12/5
Withdrawn	3/2	1/0	Missed Visit	0	0	0	0	3/1	0	6/3	0
Enrolled	47/19	14/6	Total Eligible	44/18	13/6	34/16	12/5	32/15	12/5	32/15	12/5
				100%	100%	100%	100%	90.6% / 93.3%	100%	81.3% /80.0%	100%

-There were no adverse events reported since the last continuing review report October 2011-September 2012.

-To support the submitted JMU Statement of Work, most of Year 1 of this project was dedicated to purifying and modifying antibodies to be used in a sandwich ELISA **(Figure 1)**. A summary of the work is as follows:

Figure 1. Initial Sandwich ELISA strategy. Capture monoclonal anti-lacritin antibody 1F5-C9-F4 and detector polyclonal anti-N-65 antibodies were respectively generated against lacritin's N- and C-termini.

1. All of the patient samples sent to JMU were inventoried and stored for future ELISA analysis.

2. **Figure 2** Polyclonal antibodies to the N-terminus (poly-N-term) were Protein A purified (PA-N-term).

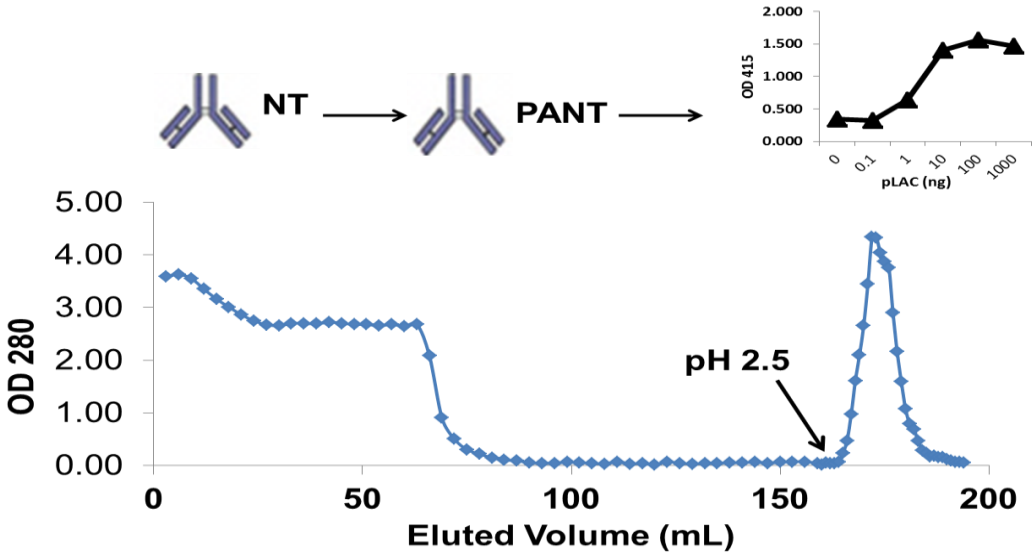


Figure 2. Protein A purified N-terminal antibody elution profile. N-terminal polyclonal antiserum was passed through a Protein A column and eluted with low pH buffer. Antibodies eluted at pH 2.5, which is represented as an increase in OD280 readings. Upper right: This antibody was then used to detect various amounts of recombinant lacritin (pLAC).

3. **Figure 3** Polyclonal antibodies to the C-terminus (poly-C-term) were Protein A purified (PA-C-term) and antigen-purified.

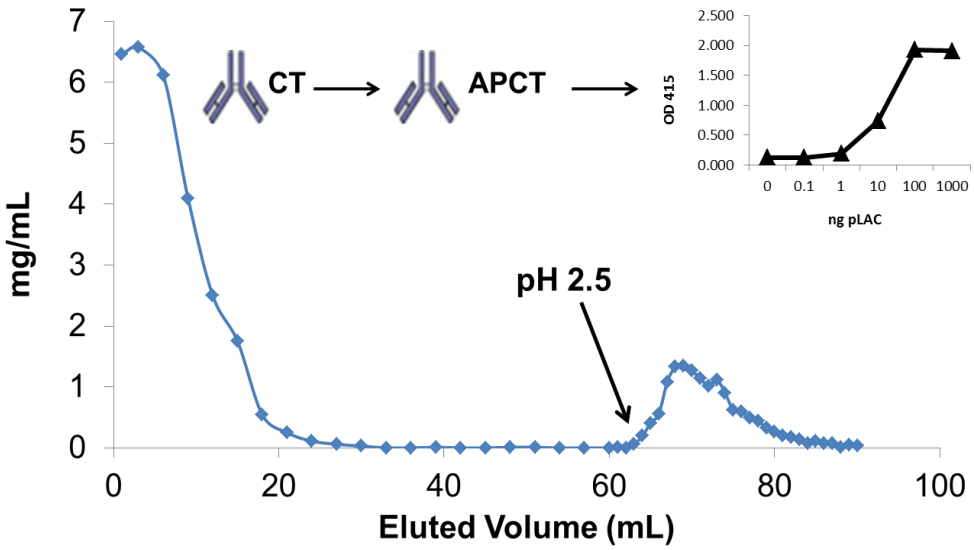


Figure 3. Elution profile of antigen-purified antibodies to the C-terminus. Polyclonal antibodies made to the C-terminus of lacritin were antigen-purified using recombinant lacritin and eluted with low pH, which is indicated by an increase in mg/ml protein. Upper right: This antibody was then used to detect various amounts of recombinant lacritin (pLAC).

4. Horse-radish peroxidase (HRP) was directly conjugated to C- and N-terminal polyclonal antibodies (HRP-N-term, HRP-C-term) and to PA-C-term (HRP-PA-C-term).

5. Polyclonal antibodies were biotinylated and used in conjunction with streptavidin (BIOT-C-term, BIOT-N-term).

6. Multiple variations of a sandwich ELISA were explored. Combinations included:

Capture Antibody	Detector antibody
Monoclonal antibody to the N-terminus	PA-C-term
Monoclonal antibody to the N-terminus	PA-N-term
Monoclonal antibody to the N-terminus	Poly-N-term
Monoclonal antibody to the N-terminus	Poly-C-term
PA-N-term	PA-C-term
PA-N-term	HRP-C-term
PA-N-term	BIOT-C-term
PA-N-term	BIOT-N-term
PA-N-term	BIOT-PA-N-term
PA-N-term	HRP-AP-C-term
PA-C-term	HRP-PA-C-term
PA-C-term	BIOT-PA-C-term
PA-C-term	BIOT-PA-N-term
PA-C-term	BIOT-AP-C-term
Poly C-term	HRP-PA-C-term

KEY

PA – protein A purified
N-term – polyclonal antiserum to N terminal peptide
C-term – polyclonal to C-terminal portion of the recombinant protein N-65
HRP – conjugated to horse-radish peroxidase
BIOT – biotinylated

KEY RESEARCH ACCOMPLISHMENTS

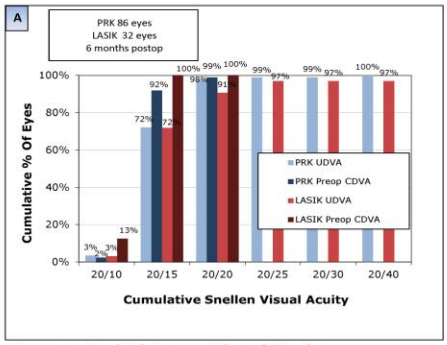
- We were unable to find a working assay using all of these combinations above. Some of the data are provided below **(Table 2)**. Similar results were observed for all combinations listed above.

  Table 2. Comparison of sandwich and indirect ELISA results for tear samples. Standard curves of both pLAC and Lac-C were generated and tear samples (WR 204 and WR 211) were analyzed for percentage in total tear protein. Antigen quantities below the limit of detection are indicated by ND (ND = Not Detected).

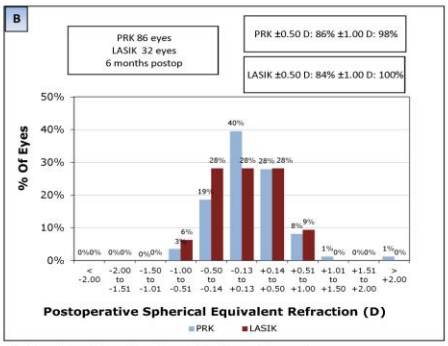
	Lacritin	
Tear Sample	**Sandwich ELISA**	**Indirect ELISA**
WR 204	**ND**	**4.8%**
WR 211	**ND**	**3.0%**

- Based on all of the work trying to find a sandwich ELISA assay that will work with tears, we are going to initially analyze all of the tear samples using the indirect method used for our normal baseline assay (**Appendix 3**: Seifert K, Gandia NC, Wilburn JK, Bower KS, Sia RK, Ryan DS, Deaton ML, Still KM, Vassilev VC, Laurie GW, McKown RL. **Tear Lacritin Levels by Age, Gender, and Time of Day in Healthy Adults.** IOVS. 2012; 53:6610-6616). Once we have an accepted sandwich ELISA working, we will analyze the samples a second time.

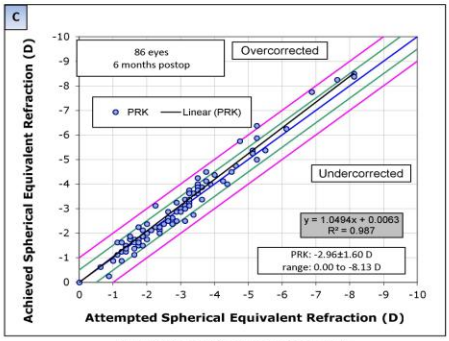
- Preliminary analysis of visual outcomes reviewing 43 patients (86 eyes) who underwent PRK and 16 patients (32 eyes) who underwent LASIK for myopia with and without astigmatism presented with the following six month postoperative results (**Figure 4. A-F**).



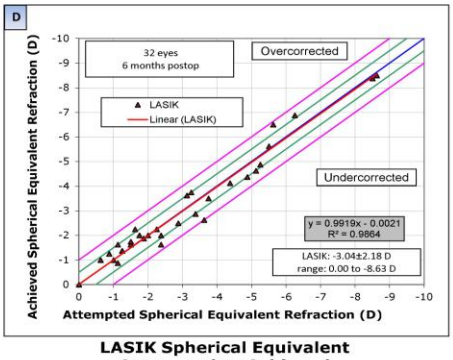
Uncorrected Distance Visual Acuity



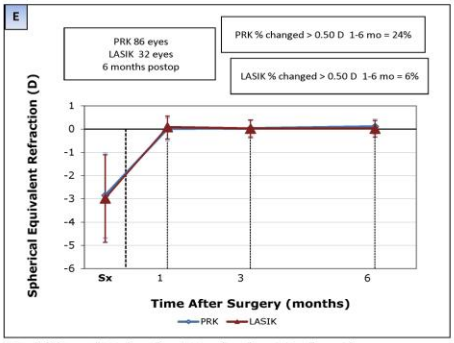
Spherical Equivalent Refractive Accuracy



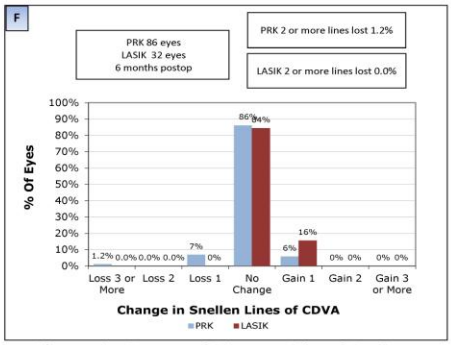
PRK Spherical Equivalent Attempted vs Achieved



LASIK Spherical Equivalent Attempted vs Achieved



Stability of Spherical Equivalent Refraction



Change in Corrected Distance Visual Acuity

REPORTABLE OUTCOMES
None

CONCLUSION
None

REFERENCES
None

SUPPORTING DATA
None

APPENDICES
Appendix 1 Current consent form, modification approval letters, and WRNMMC IRB continuing review letters 2011 and 2012.
Appendix 2 JMU approval letter 2011.
Appendix 3 Seifert K, Gandia NC, Wilburn JK, Bower KS, Sia RK, Ryan DS, Deaton ML, Still KM, Vassilev VC, Laurie GW, McKown RL. **Tear Lacritin Levels by Age, Gender, and Time of Day in Healthy Adults.** IOVS. 2012; 53:6610-6616.

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FORT BELVOIR COMMUNITY HOSPITAL (FBCH)
FORT BELVOIR, VA

This Clinical Trial consent form is valid only if it contains the IRB stamped date

Consent for Voluntary Participation in a Clinical Trial (a type of research study) Entitled: "Lacritin and heparanase levels in human tears after laser refractive surgery"

Principal Investigator: Denise S. Ryan, Ophthalmology Service, Department of Surgery (571) 231-1600

Study Sites :XX FBCH, XX WRNMMC (Walter Reed National Military Medical Center)

1. INTRODUCTION OF THE STUDY

You are being asked to participate in this study because you are an active duty U.S. military personnel and have elected to undergo either photorefractive keratectomy (PRK) or laser-assisted in situ keratomileusis (LASIK) eye surgery to correct your vision. Your participation is entirely voluntary. Refusal to participate will not result in any penalty or loss of benefits to which you are otherwise entitled, nor will your refusal affect your employment or career status.

2. PURPOSE OF THE STUDY

Although over a million laser refractive procedures are performed each year, differences in wound healing continue to cause unpredictability in outcomes and in some cases lead to complications. The human tear protein lacritin has been shown to contribute to wound healing and may improve dry eye. Lacritin activity is regulated by the enzyme heparanase (HPSE) that acts as an on/off switch for lacritin. The purpose of this study is to measure levels of lacritin and HPSE in tears of patients undergoing PRK and LASIK. Better understanding of the lacritin and HPSE response to laser refractive surgery will potentially lead to advances in wound healing and may prevent or reduce dry eye.

Other studies have shown PRK and LASIK surgery to be safe and effective in the treatment of nearsightedness, farsightedness and astigmatism (unequal curvature of the eyeball) in civilians and U.S. personnel. However, dry eye is a complication that can cause considerable problems in a small number of patients after otherwise successful surgery. Tears play a great role in eye health; they are a complex fluid containing many different compounds created in different glands and cells. Because of the many origins of tear components, it is often difficult to determine which component, if any, is involved in eye disease. To determine lacritin and HPSE's changes after refractive surgery, we will collect and test samples before and after PRK and LASIK

surgery.

The tear collection process we will be using employs a polyester fiber rod which has been shown to be a quick, non-invasive method of collecting tears.

3. PROCEDURES TO BE FOLLOWED

If you agree to be in this study, you will undergo either PRK or LASIK surgery on both of your eyes. Which surgery you have will be determined by you and your doctor. Your surgery will be done the same way as it would be done if you were not taking part in this study. You will have comprehensive eye examinations done prior to the laser surgery, 1, 3 (PRK only), and 7 days immediately after the procedure, and at 1, 3, 6, and 12 month visits postoperatively (after surgery). These appointments are "standard of care"- in other words, you would be asked to come to the clinic for these visits even if you were not taking part in this study. Information needed for your surgery and postoperative care, which is considered to be the standard of care, will be recorded during these visits for research purposes. This will include information about how well you see, and your refraction (the need for glasses), eye pressure, corneal (the clear transparent outer layer of the eye) curvature, corneal clarity, and corneal thickness.

Several eye examinations will be done specifically due to your participation in this study and are therefore being done for research purposes. These additional tests will occur at the standard visits before surgery and at the examinations done at the 1, 3, 6, and 12 months after surgery and will add an additional 30 minutes to your examination time. In addition, tear sampling will also be done at the 1 day and 1 week post-operative visit. Each of these tests has been used in clinical practice for years. They are being done for research purposes in this study so that we can attempt to find a relationship between the tear lacritin and HPSE levels, results of these tests, and any symptoms of dry eye you might experience.

The following are the additional tests at each visit:

1. Questionnaire: At each of these examinations you will also be asked to complete a questionnaire (will take about 5 minutes) for research purposes about any dry eye symptoms you may be experiencing and things that may cause you eye irritation. You will complete this questionnaire before surgery and at the 1, 3, 6 and 12 month visits after surgery.
2. Computerized corneal mapping: we will perform computerized corneal mapping to detect changes on the surface of your eye. During this test you will be seated in front of a machine that takes a picture of your eye and uses a computer program to analyze the picture. The machine doesn't touch your eye, is operated by a fully trained technician, and takes less than five minutes to complete. This will be done on both eyes before surgery and at the 1, 3, 6 and 12 month visits after surgery.
3. Tear Collection: At the eye examination done before surgery, you will be asked to undergo a tear collection procedure. If you are wearing contact lenses, you must remove

your lenses and wait 5 minutes before proceeding with the tear collection procedure. To collect your tears, a drop of 0.5% proparacaine, a local anesthetic, will be placed in the left eye. You will wait with your eyes closed for two minutes. A small polyester fiber rod will be placed in contact with the tear fluid at the corner of your eye to extract the tears for 3-5 minutes. The tear collection procedure will not hurt but may be uncomfortable. This process will be repeated on your left eye at the 1 day, 1 week, 1, 3, and 6 month post-operative appointments.
The collected tears will be sent to James Madison University (JMU) in Harrisonburg, Virginia for tear separation. Each tear sample will be split in half: the first half of each sample will be tested at JMU and the remaining half will be sent to the Rappaport Faculty of Medicine (RFM) in Haifa, Israel. These analyses will determine the profile of lacritin and HPSE in tears in response to laser refractive surgery. You do not need to take any precautions or actions prior to the collection of the tears. No personal identifying information will be sent with your tear samples to JMU or RFM. Your samples will be labeled with only a study ID number, gender and age and will not contain any part of your name or social security number. The tears collected will be destroyed in the analysis process, thus no tears are retained after completion of the assay.

4. Lissamine green stain: The doctor will examine the surface of your eye after a dye has been put in it. This test will be performed on both eyes before surgery and at the 1, 3, 6 and 12 month visits after surgery.
5. Schirmer test: This is a measurement of the amount of your tear production. You will be given an anesthetic drop (proparacaine), asked to wait 2 minutes and then fixate on an object with a slightly upward gaze and minimal blinking while a small test strip is placed on your lower eyelid. You can either keep your eyes closed gently or maintain an upward gaze with minimal blinking for 5 minutes. This test will be performed on both eyes before surgery and at the 1, 3, 6 and 12 month visits after surgery.
6. Tear break up time: We measure the time required for a dry spot to appear on your corneal surface after blinking. For this test, the surface of your eye will be touched with a small test strip containing a dye. You will be asked to blink several times to distribute the dye over your eye surface and then stare straight ahead without blinking while the doctor looks at your eye with a special light and checks how long it takes for a dry area to appear on your corneal surface. This test will be repeated five times each time it is done. This test will be performed on both eyes before surgery and at the 1, 3, 6 and 12 month visits after surgery.

The FBCH Clinic can be contacted at (571) 231-1600 and the WRNMMC clinic can be reached at (301) 295-1339.

4. AMOUNT OF TIME FOR YOU TO COMPLETE THIS STUDY

You will be part of this study for a total of one year. During this time, you will not be asked to make any extra visits to the clinic for the purposes of this study. All of the information and procedures needed for this study will be done at standard of care visits. The eye examination

done before surgery and the visits 1, 3, 6, and 12 months after surgery will take about 30 minutes longer than it would if you were not taking part in this study. The one day and 1 week visits after surgery will take 10 minutes longer than they would if you were not taking part in this study. The total amount of additional time required to participate in this study over the course of one year is approximately three hours.

5. NUMBER OF PEOPLE THAT WILL TAKE PART IN THIS STUDY

A total of up to 196 subjects are expected to take part in this study.

6. POSSIBLE RISKS OR DISCOMFORTS FROM BEING IN THIS STUDY

Any additional risks that may develop as a result of your participation in this study, other than those associated with the procedures themselves, are related to the tear collection. None of the testing procedures pose any risk beyond a normal eye examination. The following are possible risks or discomforts that may develop as a result of participation in this study:

The use of the anesthetic proparacaine may cause a mild stinging or eye irritation that may occur up to several minutes after the drop is applied. Burning, itching, pain, redness, swelling of the eye or eyelid, watering of the eyes or other irritation of eye, although rare, may also occur.

The tear collection procedure will not hurt, but may be uncomfortable. It is also possible, but very unlikely, that your eye could show a small amount of redness after the tear collection is completed. If this should happen, the redness should go away and no treatment should be needed. There may be excess tearing during the tear collection procedure. The surface of the eye could be accidentally scraped, but this would be highly unusual.

In addition to the above-mentioned risks, this study may involve risks to you that are currently unforeseeable.

While all possible risks that we know about have been listed above, other risks about which we do not know may occur or be discovered during future studies. If we find that there was a major risk to you that was not known at the time of your participation in the study, and the risk might have some effect on your health, you will be informed.

7. POSSIBLE BENEFITS FROM BEING IN THIS STUDY

You will not benefit from being in this study, but the information we learn may help us in determine how changes in lacritin and HPSE affect dry eye symptoms and wound healing after LASIK or PRK surgery.

20 August 2012

8. CONFIDENTIALITY/PRIVACY OF YOUR IDENTITY AND YOUR RESEARCH RECORDS

The principal investigator will keep records of your being in this study. These records may be looked at by people from the Walter Reed Department of Research Programs (DRP), Fort Belvoir Community Hospital DRP, the Walter Reed Institutional Review Board (IRB), and other government agencies as part of their duties. These duties include making sure that research subjects are protected. Confidentiality of your records will be protected to the extent possible under existing regulations and laws. Complete confidentiality cannot be promised, particularly for military personnel, because information bearing on your health may be required to be reported to appropriate medical or command authorities. Your name will not appear in any published paper or presentation related to this study.

A folder will be maintained containing your study records. It will include a copy of this consent form, patient information sheets, your operative report and any other related correspondence. Patient data obtained during your eye examinations before and after surgery will be recorded on worksheets and will be maintained in the folder. To protect your confidentiality, your study records will be kept in a locked file cabinet by the study coordinator at Fort Belvoir Community Hospital, Ft. Belvoir, VA with access limited to the principal investigator, research director, technical staff and study personnel. When you enter this study, you will be assigned a study ID number which will not include any part of your name or social security number. A master list will be maintained that links your study ID number with your personal identifying information. The master list will be kept in a file separate from the patient records in a locked file cabinet at Fort Belvoir Community Hospital, Ft. Belvoir, VA. Samples sent to JMU or RFM will be labeled only with your study ID number, gender and age, and not any personal identifying information. Any data sent out for analysis will be de-identified (labeled without any of your personal identifying information).

9. CONDITIONS UNDER WHICH YOUR PARTICIPATION IN THIS STUDY MAY BE STOPPED WITHOUT YOUR CONSENT

Your taking part in this study may be stopped without your consent if remaining in the study might be dangerous or harmful to you. Your taking part in this study may also be stopped without your consent if the military mission requires it, or if you become ineligible for medical care at military hospitals. The principal investigator may terminate your participation in this study if you fail to attend the before or after surgery eye examinations or elect not to undergo the laser procedure.

10. ELIGIBILITY AND PAYMENT FOR BEING IN THIS STUDY

You will not receive any payment for being in this research study.

20 August 2012

11. COMPENSATION IF INJURED AND LIMITS TO MEDICAL CARE

You will not receive any compensation (payment) should you be injured as a direct result of being in this study. You should understand that this is not a waiver or release of your legal rights. You should discuss this issue thoroughly with the principal investigator before you enroll in this study. Should you be injured as a result of your participation in this study, you will be given medical care for that injury at no cost to you.

Medical care is limited to the care normally allowed for Department of Defense health care beneficiaries (patients eligible for care at military hospitals and clinics). Necessary medical care does not include in-home care or nursing home care. If you need to be hospitalized, you may have to pay the normal fees for subsistence (hospital meals), as per standard regulations.

If at any time you believe you have suffered an injury or illness as a result of participating in this research project, and you are enrolled at WRNMMC you should contact the Department of Research Programs (DRP) at WRNMMC at 301-295-2275. If you are enrolled at FBCH you should contact Fort Belvoir Department of Research Programs at 571-231-4020.

12. COSTS THAT MAY RESULT FROM TAKING PART IN THIS STUDY

There is no charge to you for taking part in this study.

13. IF YOU DECIDE TO STOP TAKING PART IN THIS STUDY AND INSTRUCTIONS FOR STOPPING EARLY

You have the right to withdraw from this study at any time. If you decide to stop taking part in this study, you should tell the principal investigator as soon as possible; by leaving this study at any time, you in no way risk losing your right to medical care, nor will it affect your employment or career status. Some testing or period of observation by the investigators may be recommended for you in order for you to safely stop taking part in this study.

14. STEPS TAKEN BEFORE AND DURING THIS STUDY TO PROTECT YOU

To minimize the potential for increased irritation, you will be excluded from participation if you have an allergic reaction to 0.5% proparacaine or have been diagnosed with dry eyes or other surface conditions.

15. OTHER PROCEDURES OR TREATMENTS THAT YOU COULD CHOOSE

You may choose to have LASIK or PRK surgery without taking part in this study. You may also choose to have another refractive procedure done or to have a surgical alternative such as radial keratotomy or lens implants. Your doctor can provide you with more information about your nearsightedness, farsightedness and astigmatism and the benefits and risks of the different

treatments available. You are encouraged to discuss this with your doctor.

16. IMPORTANT NEW FINDINGS THAT MAY AFFECT YOUR WILLINGNESS TO STAY IN THE STUDY

If we learn new information during the study that could affect your decision to be in this study, we will tell you this information. For example, if we learn about new severe side effects of tear collection, we will tell you about these side effects. The results of the research will be provided to you if you so desire.

17. YOUR RIGHTS IF YOU TAKE PART IN THIS STUDY

Taking part in this study is your choice. You may choose either to take part or not to take part in the study. If you decide to take part in this study, you may leave the study at any time. No matter what decision you make, there will be no penalty to you and you will not lose any of your regular benefits. Leaving the study will not affect your medical care.

18. AUTHORIZATION FOR RESEARCH USE OF PROTECTED HEALTH INFORMATION

The Federal Health Insurance Portability and Accountability Act (**HIPAA**) includes a Privacy Rule that gives special safeguards to Protected Health Information (**PHI**) that is identifiable, in other words, can be directly linked to you (for example, by your name, Social Security Number, birth date, etc.). We are required to advise you how your PHI will be used.

(1) What information will be collected?

For this research study, we will be collecting information about your eye examinations (including the SOC examination), refractive surgery, ocular (eye) health status, any side effects that you are experiencing, how the treatment affects your vision, and tear analysis.

(2) Who may use your PHI within the Military Healthcare System?

The members of the research team will have access to your health information in order to find out if you qualify to participate in this study, to plan your tear collection, and to analyze the research data. Additionally, your PHI may be made available to health oversight groups such as the Walter Reed Department of Research Programs, Fort Belvoir Community Hospital Department of Research Programs, the Walter Reed Institutional Review Board, and other government agencies as part of their duties.

(3) What persons outside of the Military Healthcare System who are under the HIPAA requirements will receive your PHI?

No one outside the Military Healthcare System will receive your PHI. Data and specimens sent to the James Madison University (JMU) in Harrisonburg, Virginia and the Rappaport Faculty of Medicine (RFM) in Haifa, Israel will be labeled only with your study ID number, age and gender and not any personal identifying information.

(4) What is the purpose for using or disclosing your PHI?

We will use your protected health information during the course of the research study to: monitor your health status, measure the effects of drugs/devices/procedures on you, gather samples, determine research results, and to possibly develop new tests and procedures. The information may also be reviewed when the research study is audited for compliance.

(5) How long will the researchers keep your PHI?

The study site research team will keep the research data and the master list linking your study ID number with your personal identifying information for up to seven years after the end of the study. At the end of this time the master list will be destroyed and the research data (without any information that can link it back to you) will be kept indefinitely.

(6) Can you review your own research information?

You have the right to view your personal research information at any time during the course of the study. When the study is over, you have the right to copy your research information for your records.

(7) Can you cancel this Authorization?

Yes. If you cancel this Authorization, you will no longer be included in the research study. However, the information that has already been collected will be kept by the research team to assure patient safety. If you want to cancel your Authorization, please contact the Principal Investigator in writing.

If you decide to participate in this research study, your Authorization for this study will not expire unless you revoke or cancel it in writing to the research doctor. If you revoke your Authorization, you will also be removed from the study, but standard medical care and any other benefit to which you are entitled will not be affected in any way. Revoking your Authorization only affects the use and disclosure (sharing) of information after your written request has been received.

(8) What will happen if you decide not to grant this Authorization?

If you decide not to grant this Authorization, you will not be able to participate in this research study. Refusal to grant this Authorization will not result in any loss of medical benefits to which you are otherwise entitled, nor will your refusal affect your employment or career status.

(9) Can your PHI be disclosed to parties not included in this Authorization who are not under the HIPAA requirements?

There is a potential that your research information will be shared with another party not listed in this Authorization in order to meet legal or regulatory requirements. Examples of persons who may access your PHI include representatives of the Army Clinical Investigation Regulatory Office, the Food and Drug Administration, the Department of Health and Human Services (DHHS) Office for Human Research Protections (OHRP), and the DHHS Office for Civil Rights. This disclosure is unlikely to occur, but in that case, your health information would no longer be protected by the HIPAA Privacy Rule.

(10) Who should you contact if you have any complaints?

If you believe your privacy rights have been violated, you may file a written complaint with (if you are enrolled at WRNMMC) the Walter Reed Privacy Officer, located at 8901 Wisconsin Avenue, Bethesda, MD 20889-5600, telephone 301-319-4775 or (if you are enrolled at FBCH) the FBCH Privacy Officer, FBCH Privacy Office, located at 9300 Dewitt Loop, Oaks Pavilion, Fort Belvoir, VA 22060 at 571-231-3319.

Your signature at the end of this document acknowledges that you authorize WRNMMC/ FBCH personnel to use and disclose your Protected Health Information (PHI) collected about you for research purposes as described above.

19. CONTACTS FOR QUESTIONS ABOUT THE STUDY

If you have questions about the study, or if you think you have a study-related injury you should contact Denise Ryan at 571-231-1600. For questions about your rights as a research participant, if you are enrolled at WRNMMC contact the Walter Reed Department of Research Programs at 301-295-2275 or the Walter Reed Staff Judge Advocate Office at 301-295-2215. If you are enrolled at FBCH, contact FBCH Clinical Investigations at 571-231-4020 or the Office of the Command Staff Judge Advocate in the Sunrise Pavilion at 571-231-2877.

A copy of this signed consent form and HIPAA authorization will be provided to you.

20 August 2012

SIGNATURE OF RESEARCH SUBJECT

You have read the information in this consent form. You have been given a chance to ask questions and all of your questions have been answered to your satisfaction.

BY SIGNING THIS CONSENT FORM, YOU FREELY AGREE TO TAKE PART IN THE RESEARCH IT DESCRIBES.

_____ _____

Subject's Signature Date

_____

Subject's Printed Name

SIGNATURE OF INVESTIGATOR

You have explained the research to the volunteer and answered all of his/her questions. You believe that the volunteer subject understands the information described in this document and freely consents to participate.

_____

Investigator's Signature Date (must be the same as the participant's)

_____

Investigator's Printed Name

Version – NCR Clinical trial protocol CF&HIPAA 3June2009.doc

DEPARTMENT OF THE NAVY
WALTER REED NATIONAL MILITARY MEDICAL CENTER
8901 WISCONSIN AVENUE
BETHESDA MARYLAND 20889-5600

IN REPLY REFER TO

6500
14IV00
February 27, 2012

From: Commander, Walter Reed National Military Medical Center, Bethesda
To: Michael Mines, MD

Subj: WRNMMC IRB2 REVIEW OF 351515-16

PROJECT TITLE:	[351515-16] Lacritin and Heparanase Levels in Human Tears after Laser Refractive Surgery
REFERENCE #:	10-7243
SUBMISSION TYPE:	Amendment/Modification
ACTION:	APPROVED
APPROVAL DATE:	February 13, 2012
EXPIRATION DATE:	October 19, 2012
REVIEW TYPE:	Expedited Review

1. Thank you for your submission of Amendment/Modification materials for this research study. The WRNMMC IRB2 has APPROVED your submission. This approval is based on an appropriate risk/benefit ratio and a study design wherein the risks have been minimized. All research must be conducted in accordance with this approved submission.

2. Your request to delete LTC C. Coe, MC, USA and LTC K. Shaw, MC, USA as associate investiagators have been reviewed under the provisions of 32 CFR 219.110(b)(2) and 21 CFR 56.110 and is approved. This change to your research project will be documented in the 15 March 2012 IRB meeting minutes.

3. Be sure to maintain complete records concerning this change with your original project file.

4. You are reminded to provide all amendments, internal adverse event reports, deviations, and any other relevant information pertaining to your resesarch protocol to the Department of Research Programs through IRBNet.

5. Please do not hesitate to contact the Department of Research Programs (DRP) staff at (301) 295-8239 for any assistance or concerns.

This document has been electronically signed in accordance with all applicable regulations, and a copy is retained within our records.

DEPARTMENT OF THE NAVY
WALTER REED NATIONAL MILITARY MEDICAL CENTER
8901 WISCONSIN AVENUE
BETHESDA MARYLAND 20889-5600

IN REPLY REFER TO

6500
14IV00
October 21, 2011

From: LTC WRNMMC IRB2
To: Denise Ryan

Subj: WRNMMC IRB2 REVIEW OF 351515-15

PROJECT TITLE:	[351515-15] Lacritin and Heparanase Levels in Human Tears after Laser Refractive Surgery
REFERENCE #:	10-7243
SUBMISSION TYPE:	Continuing Review/Progress Report
ACTION:	APPROVED
APPROVAL DATE:	October 20, 2011
EXPIRATION DATE:	October 19, 2012
REVIEW TYPE:	Full Committee Review

1. The IRB reviewed the Continuing Review findings at their meeting of October 20, 2011.

2. Your research project is endorsed at More than Minimal Risk with an IRB expiration of October 19, 2012.

3. Enclosed are the stamped IRB approved consent form and HIPAA form that are to be duplicated and used to enroll new subjects. Keep each subject's original consent form and HIPAA form for each subject in your study file. A copy of the signed consent form and HIPAA form must be provided to the subject and a copy also filed in the subject's medical record.

4. You are reminded to provide all amendments, internal adverse events, deviations and any other pertinent information to the Department of Research Programs through IRBNet.

5. If you have any questions, the POC is Sheila Gaines at (301) 295-6512 or Sheila.Gaines@med.navy.mil. Please include your project title and reference number in all correspondence with this committee.

This document has been electronically signed in accordance with all applicable regulations, and a copy is retained within our records.

Institutional Review Board
WALTER REED NATIONAL MILITARY MEDICAL CENTER
8901 WISCONSIN AVENUE
BETHESDA MARYLAND 20889-5600

September 27, 2012

MEMORANDUM

From: WRNMMC IRB2
To: Denise Ryan

Subj: WRNMMC IRB2 REVIEW OF 351515-19

PROJECT TITLE:	[351515-19] Lacritin and Heparanase Levels in Human Tears after Laser Refractive Surgery
REFERENCE #:	10-7243
SUBMISSION TYPE:	Continuing Review/Progress Report
ACTION:	APPROVED
APPROVAL DATE:	September 26, 2012
EXPIRATION DATE:	October 19, 2013
REVIEW TYPE:	Full Committee Review

1. The IRB reviewed your continuing review report at their meeting on 26 September 2012. Your protocol continues to meet the requirement under 32 CFR 219.111.

2. The IRB approved, stamped consent/HIPAA authorization form is to be duplicated and used to enroll subjects. Keep the signed, original consent forms in your project file; give each subject a signed copy of the consent form.

3. You are reminded to provide all amendments, deviations, related serious adverse event, unanticipated problems involving risks to subjects or others, and any other pertinent information regarding this research protocol to the Department of Research Programs through IRBNet for reporting to the IRB.

4. You are reminded that all presentations and publications related to this work must cleared through the publications clearance process.

5. If you have any questions, the POC is Angela Davis at 301-295-2269 or angela.r.davis1@us.army.mil. Please include your project title and reference number in all correspondence with this committee.

This document has been electronically signed in accordance with all applicable regulations, and a copy is retained within our records.

From: Strong, Carolyn Denise - strongcd
Sent: Tuesday, December 06, 2011 3:35 PM
To: Seifert, Kyle - seiferkx; McKown, Robert Lee - mckownrl
Subject: IRB - (Protocol Approval)

Good Afternoon,

Thank you for delivering your signed protocol to our office today. I want to let you know that your IRB protocol entitled, "Levels of the Novel Glycoprotein Lacritin in Human Tears After Laser Refractive Surgery" is now officially approved for you to begin your study. The signed action of the board form, approval memo, and follow-up report form will be sent to you via campus mail. Your protocol has been assigned No. 12-0146. Thank you again for working with us to get your protocol approved.

As a condition of the IRB approval, your protocol is subject to annual review. Therefore, you are required to complete a follow-up report before your project end date. You must complete the follow-up report regardless of whether you intend to continue the project for another year. An electronic copy of the follow-up report form can be found on the Sponsored Programs Administration web site at the following URL:
http://www.jmu.edu/sponsprog/allforms.html#IRBform.

Your Follow-up Report must be submitted within 30 days of the project end date. Although the IRB office sends reminders, it is ultimately your responsibility to submit the continuing review report in a timely fashion to ensure there is no lapse in IRB approval.

If you have any questions, please do not hesitate to contact me.

Best Wishes,
Carolyn

****
Carolyn Strong, CIM
Research Compliance Coordinator
Sponsored Programs Administration
JMAC Bldg 6, Suite 26 MSC 5728
1031 Harrison Street
Harrisonburg, VA 22807
Phone: (540) 568-2318
Fax: (540) 568-6240
Email: strongcd@jmu.edu
****

Tear Lacritin Levels by Age, Sex, and Time of Day in Healthy Adults

Kyle Seifert,[1] *Natasha C. Gandia,*[1] *Jennifer K. Wilburn,*[1] *Kraig S. Bower,*[2] *Rose K. Sia,*[3] *Denise S. Ryan,*[3] *Michael L. Deaton,*[4] *Katherine M. Still,*[4] *Veronica C. Vassilev,*[4] *Gordon W. Laurie,*[5] *and Robert L. McKown*[4]

Purpose. Several small proteomic studies suggest that the prosecretory tear protein lacritin may be selectively downregulated in dry eye syndrome and in blepharitis, yet little information is available about normal baseline levels. This study assessed lacritin levels in tears from healthy individuals and addressed whether they differ according to sex, age, or time of day.

Methods. Rabbit antibodies against lacritin N-terminal peptide EDASSDSTGADPAQEAGTS (Pep Lac N-Term) were generated and characterized against human recombinant lacritin and N-65 truncation mutant. Basal tears were collected from 66 healthy individuals ranging in age from 18 to 52 years, and at four times during one 24-hour period from 34 other individuals. Lacritin levels were then analyzed by ELISA and Western blotting.

Results. Anti-Pep Lac N-Term bound lacritin, but not truncation mutant N-65 that lacks the N-terminal antigenic site. Tear lacritin levels followed a normal distribution with a mean of 4.2 ± 1.17 ng/100 ng total tear protein. Levels differed little by age or sex, and decreased slightly between 4 and 8 hours in a 24-hour cycle. Tear-blocking effects were minimal, as suggested by spiking of tears with recombinant lacritin.

Conclusions. Anti-Pep Lac N-Term–detectable lacritin comprises ~4.2 ng/100 ng total tear protein in healthy individuals, with no significant differences between males and females or among individuals between 18 and 52 years old. Levels decrease slightly in the late afternoon. These findings provide a baseline for future immunodiagnostic studies of lacritin in dry eye and other ocular diseases. (*Invest Ophthalmol Vis Sci.* 2012;53:6610–6616) DOI:10.1167/iovs.11-8729

From the departments of [1]Biology and [4]Integrated Science and Technology, James Madison University, Harrisonburg, Virginia; and the [2]Wilmer Eye Institute, Johns Hopkins University, Baltimore, Maryland; the [3]Center for Refractive Surgery, Walter Reed Army Medical Center, Washington, DC; and the [5]Department of Cell Biology, University of Virginia, Charlottesville, Virginia.

Supported by grant funding from Virginia's Commonwealth Health Research Board (KS and RLM), NIH RO1 EY013143, and NIH RO1 EY018222 (GWL).

Submitted for publication October 3, 2011; revised June 1 and July 11, 2012; accepted August 18, 2012.

Disclosure: **K. Seifert**, None; **N.C. Gandia**, None; **J.K. Wilburn**, None; **K.S. Bower**, None; **R.K. Sia**, None; **D.S. Ryan**, None; **M.L. Deaton**, None; **K.M. Still**, None; **V.C. Vassilev**, None; **G.W. Laurie**, EyeRx Research, Inc. (F), P; **R.L. McKown**, EyeRx Research, Inc. (F), P

Corresponding author: Robert L. McKown, Department of Integrated Science and Technology, James Madison University, 701 Carrier Drive, Harrisonburg, VA 22807; mckownrl@jmu.edu.

Lacritin is a 12.3 kDa human tear glycoprotein that is prosecretory, mitogenic,[1] and antimicrobial (McKown RL, et al. *IOVS* 2010;51:ARVO E-Abstract 4181) and that promotes sustained basal tearing in rabbits.[2] Lacritin acutely augments constitutive but not stimulated lacrimal acinar cell secretion, even when prior treatment with interferon-γ and TNF has neutralized the response to carbachol (Fujii, et al. *IOVS* 2011;52:ARVO E-Abstract 3714).

Lacritin's ability to stimulate tear production makes it an interesting protein to study for its potential involvement in dry eye syndrome and other eye-related diseases. Dry eye affects the lives of over 25 million Americans, yet it is poorly understood and lacks sensitive early-stage diagnostics. Current tests are more appropriate for later disease stages, making difficult the diagnosis of patients with mild to moderate symptoms.[3] Moreover, tests such as Schirmer strips, ocular surface staining, and tear film breakup time are still not uniformly applied[4] (although standardization has improved with publication of the International Dry Eye Workshop report[5]), and new devices to assess tear osmolarity show promise,[6] although not in isolation.[7] Development of an assay to help diagnose both early-onset and later dry eye, recognizing that there may be different etiologies, would be of great benefit.

Dry eye syndrome and other associated conditions are believed to correlate with changes in specific protein content of the ocular surface.[8] Some small proteomic studies suggest that lacritin is one of only 4% to 5% of the tear proteome that is downregulated in dry eye or dry eye–related conditions.[9] Lacritin levels measured by mass spectroscopy analysis of tear samples were 7-fold less from 11 individuals with contact lens–related dry eye than from 10 users of contact lenses with normal eye conditions.[10] Sensitivity, inability to provide relative tear concentrations, and lack of information on normal baseline levels or whether lacritin levels are subject to time-of-day variation are all limitations of these studies. Blepharitis is characterized by inflammation of the eyelid and dry eye conditions. A study using electrospray-quadrupole-time-of-flight mass spectrometry (ESI-Q-TOF) identified several changes in tear proteins.[11] Lacritin was one of nine proteins downregulated by ~50%.[11]

Quantitation of major tear proteins has been studied by gel electrophoresis (lactoferrin, tear-specific prealbumin, and lysozyme)[12–14] and by size-exclusion chromatography combined with enzymatic assays and immunologic methods (lysozyme, IgA, IgG, albumin, and lactoferrin).[15] More recent studies have employed a variety of mass spectrometry–based methods.[16–21] Antibody-based methods have also been used to quantify tear proteins, including sandwich ELISA[22] or sandwich ELISA following size-exclusion high-performance liquid chromatography to assess levels of several major tear proteins.[23,24] Membrane arrays have comprehensively docu-

mented tear cytokines,[25,26] and a new microfluidic chip approach shows promise.[27]

To date, no assay has been developed to quantitate tear lacritin levels. In this study we established an indirect lacritin ELISA that is based on the new N-terminal-specific anti-lacritin antibody (anti-Pep Lac N-Term) and screened normal basal tear samples from 66 healthy individuals of different ages and both sexes. Tears were also collected four times during a 24-hour period from 34 others to assess time-of-day variation. This analysis of lacritin in normal healthy individuals sets the stage for future analysis of lacritin in dry eye.

Methods

Lacritin Peptide, Anti-N-terminal Anti-lacritin Antisera, and Recombinant Lacritins

Peptide EDASSDSTGADPAQEAGTS (Pep Lac N-Term), corresponding to the N-terminus of mature human lacritin (amino acids 1–19 without signal peptide), was synthesized (>85% purity) and conjugated to keyhole limpet hemocyanin (KLH) by Bio-Synthesis, Inc. (Lewisville, TX). New Zealand white rabbits were immunized in three boosts with Pep Lac N-Term-KLH. Final antiserum (anti-Pep Lac N-Term) was collected on day 70. Preimmune serum was collected before immunization (Bio-Synthesis, Inc.).

Recombinant human lacritin was generated and purified from the lacritin–intein fusion plasmid pLAC.[28] Lacritin lacking 65 amino acids from the N-terminus (N-65) (McKown RL, et al. *IOVS* 2010;51:ARVO E-Abstract 4181) was generated out of pLAC using the forward primer 5′-GGGAATTCCATATGAAATCCATAGTGGAGAAAAGT-′3 and reverse primer 5′-GGGAATTCCATATGTATATCTCCTTCTTAAAG3-3′. Recombinant proteins were expressed in *E. coli*, affinity purified on chitin beads (New England BioLabs, Inc., Ipswich, MA), eluted without intein tag,[28] and then further purified on DEAE-Sepharose (GE Healthcare, Little Chalfont, UK), as previously described.[2] Purified proteins were freeze-dried and stored at −80°C until use.

Enzyme-Linked Immunsorbent Assay

To assess anti-Pep Lac N-Term specificity, plates were coated overnight with 100 μL lacritin or N-65 diluted 0, 50, or 100 ng/mL in coating buffer (0.017 M $NaHCO_3$, 0.015 M Na_2CO_3, pH 9.6).[29] For assay of tear samples, 100 ng total tear protein was coated in each well. To generate a standard curve of recombinant lacritin, each plate contained triplicate wells to which was added 0, 0.5, 1, 2, 4, 6, or 8 ng protein. Wells were washed, blocked with PBS-Tween (PBS with 0.3% Tween-20 [PBS-T]), and then incubated for 1 hour at 37°C with 100 μL anti-Pep Lac N-Term antiserum or preimmune serum diluted 1:200 in PBS-T. After washing three times with PBS-T, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (MP Biomedicals, Solon, OH) diluted 1:1,000 in PBS-T was added for 1 hour (37°C). Plates were washed three times with PBS-T, and then bound antibody was measured after incubation for 10 minutes with 100 μL OPD substrate (Acros Organics, Geel, Belgium) by absorbance at 415 nm (model 680; Bio-Rad, Hercules, CA). The same ELISA protocol was used for determining human tear lysozyme concentrations with lysozyme from human milk (Sigma-Aldrich, St. Louis, MO) for the standard curve and rabbit anti-human lysozyme polyclonal antibodies (MP Biomedicals) diluted 1:200 in PBS-T for detection.

Western Blot

Recombinant lacritin, N-65, or tear samples were loaded on 4% to 20% Mini-PROTEAN TGX precast gels (Bio-Rad), electrophoresed at 200 V, and transferred to nitrocellulose (Protran BA 83; Whatman, Dassel, Germany). Blots were blocked with PBS-T, incubated with anti-Lac Pep N-Term (1:500 dilution in PBS-T) for 2 hours at room temperature, rinsed with PBS-T, and incubated for 2 hours at room temperature with HRP-conjugated goat anti-rabbit IgG (MP Biomedicals) diluted 1:10,000 in PBS-T. Blots were rinsed with PBS-T and developed via chemiluminescence with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific Inc., Rockford, IL).

Tear Collection

Tear collection followed the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board at Walter Reed Army Medical Center, where tears were collected, and at James Madison University, where the tears were analyzed. Sixty-six subjects aged 18 to 52 years voluntarily enrolled after explanation of the nature and potential risks of the study. Subjects wearing contact lenses were instructed to remove their lenses and wait at least 5 minutes before proceeding with tear collection. After one drop of 0.5% proparacaine was instilled on the ocular surface (Lossen VI, et al. *IOVS* 2010;51:ARVO E-Abstract 6279), excess fluid around the eye was blotted dry, and patients were instructed to sit with eyes gently closed for 2 minutes. Tears were collected from the lower cul-de-sac of the left eye into a 2 × 10 mm polyester fiber rod (Transorb Wick; Filtrona, Richmond, VA), as previously described.[30–32] Collection was for a time sufficient to obtain fluid without irritation (3–5 minutes per consent form; actual time 1–2 minutes). Each wick with tears was placed into a modified micropipette tip in a 1.5 mL Eppendorf tube for −70°C storage. Tears were subsequently eluted from wicks with 30 μL PBS per wick for 20 minutes, followed by centrifugation for 10 minutes at 16.2*g*. Eluted tear samples were stored at −70°C. Protein concentration was assessed by the BCA assay (Thermo Scientific BCA Protein Assay Kit; Pierce Biotechnology, Rockford, IL) using bovine serum albumin as a protein standard.

Statistical Analysis

All tear samples were assayed in triplicate on each of two or three separate plates. Statistical analytical details have been published previously (see Supplemental Methods, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8729/-/DCSupplemental).

Results

Purification of Recombinant Lacritins and Characterization of Anti-Pep Lac N-Term

Tear lacritin is a 119 amino acid glycoprotein with several alpha helices, including a C-terminal amphipathic alpha helix.[28] Truncation mutant N-65 lacks 65 amino acids from the N-terminus (Fig. 1). Recombinant lacritin and N-65 were generated as intein fusion proteins that make possible purification on chitin beads and on column release of each from the intein tag. Release yields two predominant Coomassie blue–stainable bands of 18 and 6 kDa, plus a minor band of 75 kDa (Fig. 1B, lane 2). Further purification on DEAE-Sepharose produced a single protein band of 18 kDa (Fig. 1B, lane 4) that has been confirmed as lacritin by mass spectroscopy analysis (data not shown). Intact recombinant lacritin was the antigen for the first anti-lacritin polyclonal antibody that was suitable for immunolocalization and ELISA, but not for Western blotting.[1] Subsequent discovery of alternative splice variants affecting the C-terminus[9,33] presented a need for domain-specific antibodies capable of monitoring lacritin levels and lacritin integrity in normal and dry eye tears. Rabbits were immunized with synthetic peptide (Pep Lac N-Term-KLH) corresponding to the first 19 amino acids of tear lacritin, a region lacking from N-65 (Fig. 1A). Increasing amounts of lacritin or N-65 were adsorbed to ELISA plates or separated by SDS-PAGE and transferred to nitrocellulose. Anti-Pep Lac N-Term detected lacritin, but not N-

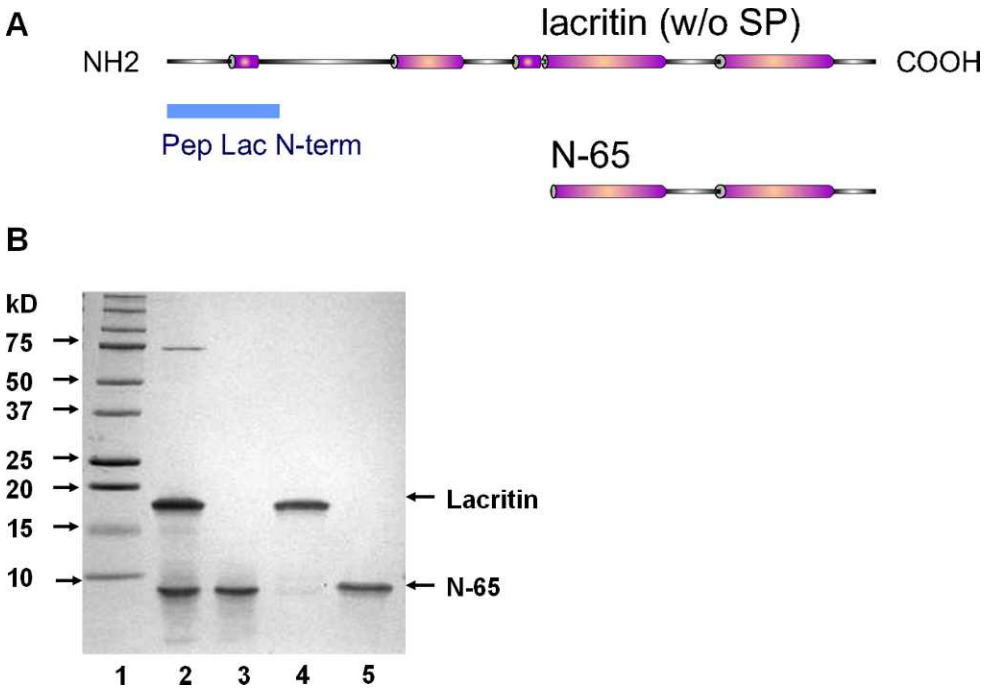

Figure 1. Purification of recombinant lacritin and deletion variant N-65. (**A**) Diagram of lacritin without signal peptide shown with boxed regions of predicted alpha-helices, and N-65 truncation mutant lacking 65 amino acids from the N-terminus. Also shown is the location of the Pep Lac N-term antigen. (**B**) SDS-PAGE with Coomassie stain: *lane 1*, molecular weight standards; *lane 2*, eluant from chitin affinity chromatography of lacritin; *lane 3*, DEAE-Sepharose 14 mM NaCl flow-through lacritin; *lane 4*, DEAE-Sepharose 140 mM NaCl step elution of lacritin; *lane 5*, DEAE-Sepharose–purified N-65.

65, at coating levels of 5 and 10 ng (Fig. 2A), and in blots of 100, 200, or 400 ng lacritin but not of N-65 (Fig. 2B). No lacritin or N-65 titer was detected in the preimmune serum (data not shown). Thus anti-Pep Lac N-Term displays N-terminal domain specificity and appears to be highly sensitive.

Lacritin Tear Analysis

ELISA plate-to-plate variability is controllable by the inclusion of internal standards. Accordingly, each tear-coated plate included wells of 0.5 to 8 ng added lacritin from which a standard curve could be generated (Fig. 3A). R^2 values were required to exceed 0.97[34] and calculated lacritin tear values to have at least two standard curve data points on either side of the calculated value. Plates and samples that did not meet the required criteria were removed from the population. These criteria reduced the number of tear samples reported from 66 to 58. The capacity of tear lacritin to compete with other tear proteins for adsorption to microwell plastic[25] was a concern. We chose a coating level per well of only 100 ng total protein of tears, a level in keeping with 70% to 80% coating efficiency[35] according to a binding capacity of ~154 ng/well. This coating level was also appropriate for the small volume of collectable tears per individual. Nonetheless, it was possible that lacritin's nonpolar side chains might not be available for hydrophobic adsorption to microwell plastic. To address this issue, increasing amounts of recombinant lacritin were spiked into recombinant lacritin or into human tears (Fig. 3B). Detection of lacritin by ELISA increased linearly when recombinant lacritin was added to either recombinant lacritin or tears with a fixed concentration of lacritin, suggesting that tear lacritin is likely adsorbed to microwell plastic with minimal interference from other tear constituents.

We next compared lacritin levels in normal human tears using in-plate standard curves to express values as nanograms of lacritin per 100 ng total tear protein or as percent lacritin. A minimum of six determinations for each individual (three wells per plate from two plates) were used for statistical analysis. Tear lacritin values for the population of normal and relatively young individuals in this study followed a normal distribution

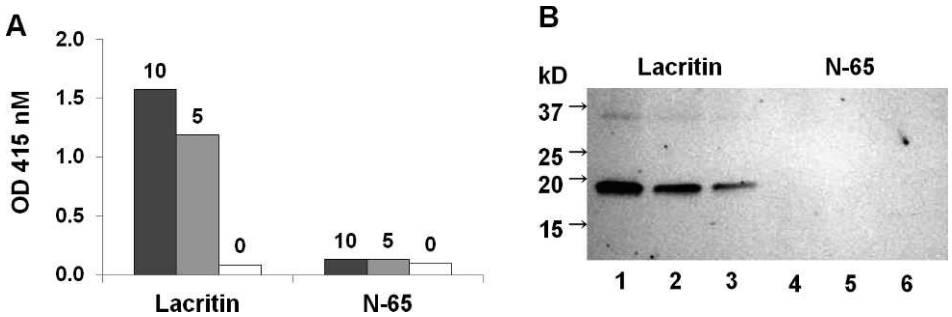

Figure 2. Specificity of anti-Pep Lac N-Term antibodies for full-length lacritin, and lack of reactivity with N-65. (**A**) ELISA of anti-Pep Lac N-Term antibodies against 0, 5, and 10 ng of lacritin or N-65. (**B**) Western blot of decreasing amounts of purified lacritin and N-65 incubated with anti-Pep Lac N-Term antibodies and developed via chemiluminescence: *lanes 1 and 4*, 400 ng; *lanes 2 and 5*, 200 ng; *lanes 3 and 6*, 100 ng.

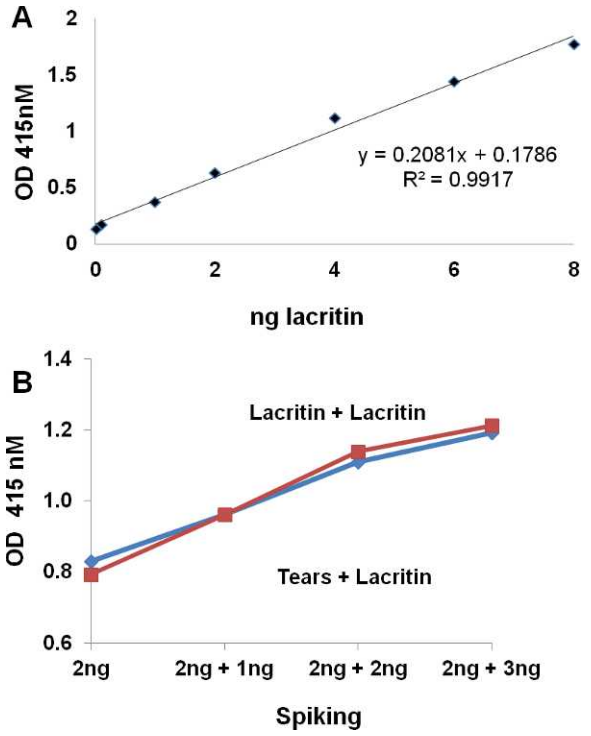

Figure 3. ELISA standard curve and spiking experiments. (**A**) Example of a lacritin standard curve replicated in each ELISA plate. (**B**) Two spiking experiments. In one of these, increasing amounts of lacritin were added to a human tear sample with an observed lacritin concentration of 2 ng. In the second experiment, increasing amounts of lacritin were added to 2 ng lacritin. In both experiments, mixtures were analyzed by ELISA.

with a mean of 4.2 ± 1.17 ng/100 ng total tear protein (Fig. 4). Little apparent difference between the sexes was detected (Table). Note that the standard deviation values reported here are not estimates of variation in the corresponding population, as they include variations attributed to the number of replicates and plates used on each patient's sample. Moreover, the number of males and females tested (Table) was not the same, and their ages were not evenly distributed from the youngest (18 years) to the oldest (52 years). In order to better visualize the distribution of percent lacritin as a function of age and sex, a scatter plot of all 58 individuals was generated (Fig. 5). This analysis confirms a lack of apparent difference by age or sex within this population.

Percentile	%Lacritin
99.0%	6.9
97.5%	6.4
95.0%	6.1
90.0%	5.7
80.0%	5.2
70.0%	4.8
60.0%	4.5
50.0%	4.2
40.0%	3.9
30.0%	3.6
20.0%	3.2
10.0%	2.7
5.0%	2.3
2.5%	2.0
1.0%	1.6

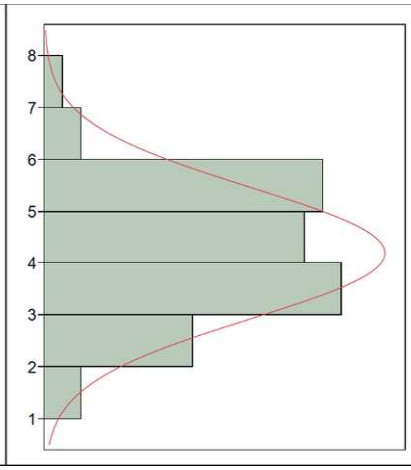

Figure 4. Normal distribution fit and normal distribution–based percentiles for percent lacritin (ng lacritin/100 ng total tear protein) in tears of a healthy population. Percentile values are given to indicate ranges of patient-wise lacritin values that might be considered unusual with respect to the population represented in this study.

Table. Lacritin in Normal Human Tears

	Male	Female	Combined
Number	43	15	58
Average age, y	32	33	33
Average lacritin*	4.11	4.47	4.20
Standard deviation	1.31	1.13	1.26
Maximum	7.52	6.85	7.52
Minimum	1.33	2.99	1.33

* Average lacritin = ng lacritin/100 ng total tear protein.

Detection of Tear Lacritin by Western Blot

Lacritin pre-mRNA is subject to alternative splicing.[33] Once synthesized, 12 O- and 1 N-glycoslyation sites are predicted.[9] In Coomasie blue staining of recombinant lacritin (Fig. 1B), the appearance of a ~6 kDa lacritin proteolytic fragment was noted. In Western blots of seven equally loaded tear samples, anti-Pep Lac N-Term detected a prominent 20 to 25 kDa band of variable width and intensity (Fig. 6). The breadth of the 20 to 25 kDa band is in keeping with O- and N-glycosylation[1,9] of 4 to 7 kDa, as calculated versus the 18 kDa mobility of unglycosylated bacterial recombinant lacritin.

Comparison to Tear Lysozyme

Lysozyme is a prominent tear component that serves as a useful comparative benchmark. Using the same ELISA approach and purchased human lysozyme and anti-human lysozyme antibodies, we assessed tear lysozyme levels in selected samples. Tear lysozyme ranged from 18 to 23 ng lysozyme/100 ng total tear protein with an average of ~20 ng lysozyme/100 ng total tear protein. Therefore the concentration of human tear lacritin can be estimated to be approximately one-fifth the concentration of human tear lysozyme by this analysis. Figure 7 shows an example of this analysis with two standard curves from which the concentration of lacritin was calculated at 3.6 ng lacritin/100 ng total tear protein and that of lysozyme at 22 ng lysozyme/100 ng total tear protein for the tear sample shown.

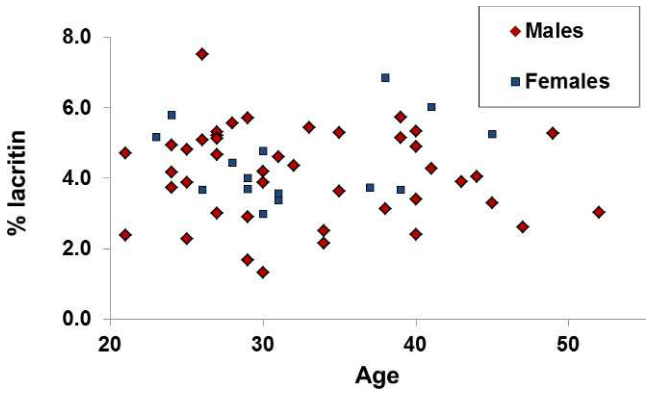

Figure 5. Distribution of percent lacritin (ng lacritin/100 ng total tear protein) for males and females by age. Percent lacritin was determined for 58 individuals (43 males and 15 females) and plotted as a function of age (18–52 years).

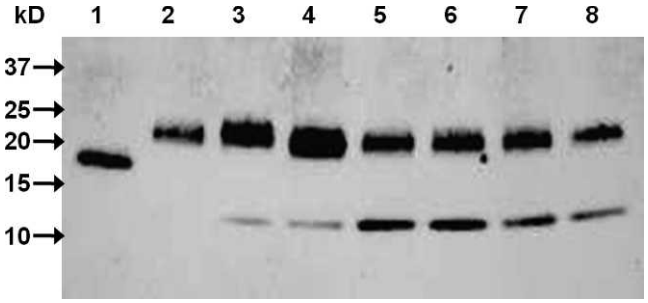

FIGURE 6. Western blot analysis of selected tear samples and recombinant lacritin. Here 200 ng of purified recombinant lacritin (*lane 1*) and 2 μg total protein of tear samples from seven individuals (*lanes 2–7*) were analyzed by Western blot. Blots were incubated with anti-Pep Lac N-Term antibodies and developed by chemiluminescence. Recombinant lacritin made in *E. coli* (*lane 1*) is not glycosylated, while human tear lacritin is glycosylated (*lanes 2–7*).

Time-of-Day Study

Some tear proteins vary in amount over the daily cycle.[14,36] To determine if the same is true of lacritin, samples were collected from 34 additional individuals at time 0 hours (7:30–8:30 AM), 4 h (11:30 AM to 12:30 PM), 8 h (4:30–5:30 PM), and 24 hours (7:30–8:30 AM the following day) and assayed for lacritin (Fig. 8). Nested, mixed ANOVA points to significant differences across time ($P = 0.0341$) that by Tukey's honestly significant difference (HSD) test may be represented by a slight decrease between 4 and 8 hours.

DISCUSSION

We report that anti-Pep Lac N-Term antibody specifically binds prosecretory mitogen lacritin in basal human tears with minimal apparent blocking effect and that detected levels vary little by age, sex, or time of day. In most normal individuals, lacritin appears to represent 4.2 ng/100 ng total tear proteins by indirect ELISA, suggesting that lacritin may be at micromolar levels in tears. The range for percentage total protein for lysozyme from this analysis is consistent with previously published concentrations of tear lysozyme.[37]

Although levels varied little by age, samples were acquired from a relatively young group of normal individuals. Basal tear peroxidase decreases during menopause in women and around age 80 in men.[38] Similarly, PLA2G2A is significantly decreased in basal tears of 60- to 70-year-old women and 70-year-old men.[39] An earlier study of reflex tears from 20- to 82-year-olds, not distinguished by sex, reported a negative correlation of lysozyme and lactoferrin by age, whereas IgG and ceruloplasmin increased with age.[22] Thus tears from older women and men would be helpful for more thoroughly investigating the effect of age on lacritin levels.

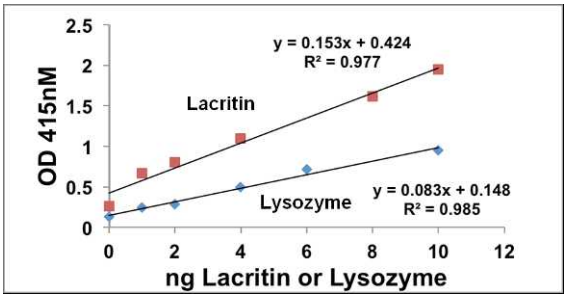

FIGURE 7. Example of standard curves for recombinant lacritin and human lysozyme used to determine protein concentrations in tear samples. Increasing concentrations of lacritin and human lysozyme were analyzed by ELISA with anti-Pep Lac N-Term and anti-human lysozyme, respectively. The concentration of lacritin was calculated at 3.6% of total protein and that of lysozyme at 22% of total protein for the same human tear sample.

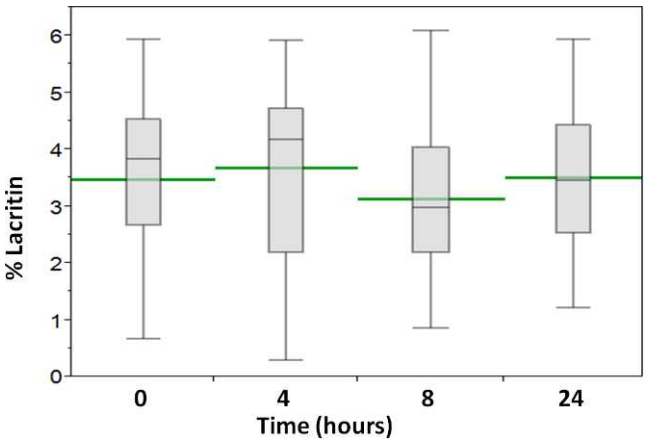

FIGURE 8. Box plots for the percent lacritin averages over 24 hours. Each "*box*" represents the range covered by the middle 50% of individuals. The *horizontal lines* within each *box* represent the median value in each group, and the *long horizontal lines* that cross each *box* represent the mean value for each group.

The issue of tear protein in males versus females was recently queried in a proteomic study of reflex tears from 20 normal male and female individuals pooled by sex.[16] Lacritin, lipocalin, haptoglobin, mammoglobin B precursor, cystatin S precursor, and anti-alpha 1 trypsinogen were all reported to be greater in female tears. Additional studies with larger numbers of females, in particular tears from older women, would be helpful for more thorough investigation of the effect of age on tear lacritin levels.

The time-of-day variation of major tear proteins has been investigated.[14,36] We also looked at diurnal variation of lacritin over a 24-hour period. In that study, lacritin levels dropped slightly in late afternoon, but the change was minimal, so sampling time from patients would not be crucial for comparison with other tear samples or for lacritin determination. Some studies have demonstrated changes in total protein and lacrimal secretion when samples were taken from closed versus open eyes.[26,40] All of the samples in the current study were collected from open eyes. Additional studies could also investigate the amount of lacritin in closed eye samples.

Quantitative detection of a single protein in a complex fluid is optimally performed by sandwich ELISA or membrane array, in which an optimized coat of immobilized antibody captures a target protein for detection by a second antibody directed to a different epitope. An assay with known amounts of purified target protein then establishes a standard curve for extrapolation of target protein in complex fluids. Such an approach has been successfully employed to detect picogram/milliliter levels of angiogenin (ANG), epidermal growth factor (EGF), chemokine (C-X-C motif) ligand 1 (CXCL1), chemokine (C-X-C motif) ligand 5 (CXCL5), chemokine (C-X-C motif) ligand 10 (CXCL10), interleukin 8 (IL-8), chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-C motif) ligand 4 (CCL4), insulin-like growth factor binding protein 2 (IGFBP2), TIMP metallopeptidase inhibitor (TIMP-1), and TIMP metallopeptidase inhibitor 2 (TIMP-2) in normal reflex tears.[26] Although anti-Pep Lac N-Term was originally envisioned as the capturing antibody in a sandwich ELISA, generating a detector antibody has been

challenging through four separate attempts in rabbits and mice; however, the linearity of detection when increasing amounts of recombinant lacritin were spiked into whole tears implied that tear blocking was minimal and that the assay was sensitive. Moreover, the subsaturating quantity of tears added was appropriate for 70% to 80% adsorption.[35]

Subsequent studies will analyze lacritin levels in tears of dry eye, Sjögren's syndrome, and pre- and post-photorefractive keratectomy (PRK) surgery individuals as a potential new diagnostic for dry eye.

Acknowledgments

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